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Extensive cross-reactive neutralizing antibody response in Indian patients with limited genetic diversity of HIV-1

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Abstract

Genome sequence analysis of HIV-1 subtype C viruses from India shows monophyletic lineage and relatively limited genetic diversity. To understand its immunological implication, cross-reactivity of neutralizing antibody response was investigated. In primary screening, neutralizing antibody response to single heterologous primary HIV-1 subtype C isolate was assessed in plasma samples from 235 HIV-1 infected, antiretroviral treatment naive individuals from Pune, India. Plasma samples that showed ≥90% neutralization and two randomly selected plasma samples that showed 50–60% neutralization were tested against a panel of primary HIV-1 subtype C isolates obtained from epidemiologically unlinked individuals from India. The neutralizing antibody response showed extensive cross-neutralization, suggesting presence of shared neutralization determinants among circulating HIV-1 subtype C viruses in India.

Keywords: HIV subtype C; India; Cross-neutralization; Genetic diversity

Introduction

HIV-1 subtype C has several unique properties such as predominance of R5 phenotype even in patients with advanced disease (Cecilia et al., 2000), relatively conserved V3 loop (Gaschen et al., 2002), three Nuclear Factor (NF)-κB binding sites in Long Terminal Repeats (LTR) (Choudhury et al., 2000) and resistance to neutralization by human monoclonal antibodies 2G12 and 2F5 that show broad neutralizing activity against HIV-1 non-subtype C isolates (Binley et al., 2004). Shankarappa et al. (2001) reported that HIV-1 subtype C nucleotide sequences of V3–V4 region of *env* from India were closely related to each other. Our studies on HIV-1 *env* (gp120 and gp41), *gag* and *nef* have also shown that HIV-1 subtype C nucleotide sequences from India form monophyletic lineage (Agnihotri et al., 2004, 2006; Jere et al., 2004; Kurle et al., 2004). Similar results were obtained by analysis of available full-length genome sequences from India (Novitsky et al., 1999, 2002). These observations point towards limited genetic diversity among Indian HIV-1 subtype C viruses.

Limited genetic diversity may have important immunological advantage. There may be greater sharing of epitopes among the circulating viruses. Therefore, immune response induced against one strain may extensively cross-react with the other strains. In a study of HIV-1 subtype C infected individuals from South Africa (Bures et al., 2002), broadly cross-reactive neutralizing antibodies were detected along with neutralizing antibody response to autologous virus. Based on this observation, the authors suggested that HIV-1 vaccine might have to overcome less epitope diversity in South Africa.

Studies of HIV-1 specific Cytotoxic T Lymphocyte (CTL) response in Indian patients (Paranjape et al., 1998; Thakar et al., 2002) showed cross-reactive CTL response and presence of conserved immunodominant regions in HIV-1 subtype C Gag and Nef (Thakar et al., 2005). This finding is consistent with the hypothesis that there is extensive sharing of epitopes within HIV-1 subtype C viruses in India. Generating such data is likely to be important in the context of development of prophylactic HIV-1 vaccine. However, there is paucity of data

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on characteristics of neutralizing antibody response generated in HIV-1 subtype C infected individuals from India.

We investigated neutralizing antibody response developed by HIV-1 infected individuals against multiple heterologous subtype C isolates obtained from epidemiologically unlinked patients from India.

Results

Primary screening for HIV-1 neutralizing activity of plasma derived from HIV-1 seropositive individuals

Plasma samples from 235 HIV-1 seropositive individuals obtained during years 2001 to 2003 from Pune (Maharashtra), India were tested against a heterologous, primary HIV-1 subtype C isolate (VB 49). The VB 49 was isolated from HIV-1 seropositive individual from Pune, India in year 1999. All plasma samples were tested at 1/10 dilution for HIV-1 neutralizing activity using GHOST cell assay as described earlier (Trkola et al., 1998). Out of 235 samples, 19 (8%) showed ≥90% neutralization (≥1 log reduction in infectivity) of VB 49, whereas 51 (21%) showed neutralization in the range of 50 to 89%. Remaining samples showed <50% neutralization.

Background response shown by 20 plasma samples from healthy, HIV seronegative individuals at this dilution was 5.8±8.3% (mean±SD).

Neutralizing activity was not found to be associated with $CD4^+$ T cell count, plasma viral RNA level and clinical status (data not shown).

Neutralizing antibody response showed extensive cross-reactivity against heterologous viruses isolated from epidemiologically unlinked individuals

Nineteen plasma samples that showed ≥90% neutralization in primary screening were tested against a panel of 13 primary HIV-1 isolates (including the virus isolate used in primary screening). The isolates were obtained from epidemiologically unlinked (as per our knowledge) individuals residing in four different states of India and were isolated between year 1999 and 2005 (Table 1). Serial two-fold dilutions of each plasma sample were tested against each HIV-1 isolate for neutralizing activity using GHOST cell assay.

Extensive cross-neutralization of heterologous viruses suggesting presence of shared neutralization determinants among viruses circulating in India was observed (Table 1). Table 1 presents highest plasma dilution that showed 50% neutralization (ID_{50}) of thirteen virus isolates from the test panel. We have also shown plasma-virus reactions where ≥90% neutralization was observed (Table 1) as ≥90% neutralization is likely to have more biological relevance (Mascola, 2003; Nishimura et al., 2002). The negative control (PNP: Pooled Normal Plasma from 12 healthy, HIV seronegative individuals) tested at 1/10 dilution showed <30% background response in all the assays.

In Table 1, we have organized viruses in decreasing order of neutralization sensitivity (based on geometric mean ID_{50} shown against all plasma samples) and plasma samples in decreasing order of neutralization capacity (based on geometric mean ID_{50} against all HIV-1 isolates). Geometric mean ID_{50} against 19 plasmas ranged between 17.7 for BH 9390 and 160.0 for VB 39. Conversely geometric mean ID_{50} against 13 viruses ranged from 14.1 for CPI 218 to 201.6 for CPI 177.

Out of 19 plasma samples, 7 samples (CPI 177, 220, 267, 243, 287, 255, 261) showed ≥90% neutralization against majority of isolates (≥11) and ≥50% neutralization against all isolates from test panel. Eight plasma samples (CPI 229, 91, 244, 294, 223, 212, 133, 214) showed ≥90% neutralization of 5 to 10 isolates and ≥50% neutralization against >10 isolates from test panel. Three plasma samples (CPI 158, 153, 99) showed ≥90% neutralization of 1 to 3 isolates and ≥50% neutralization against 7 to 11 isolates from test panel. Remaining plasma sample (CPI 218) showed ≥50% neutralization against four isolates (Table 1).

Since there are multiple neutralization determinants for HIV-1, heterologous viruses that are more close to autologous virus may be neutralized at higher titer compared to more distant viruses. This may be evidenced by ID_{50} value against given isolate. Comparison of the ID_{50} values shown by the 19 plasma samples against virus isolates from Maharashtra (state from where all plasma samples were obtained) and virus isolates obtained from other states (Karnataka, Goa, Delhi) showed that cross-neutralization was irrespective of origin of viruses.

Additionally two plasma samples (CPI 129 and CPI 194) that showed 50–60% neutralization in primary screening (against isolate VB 49) were randomly selected and tested against panel of 8 primary HIV-1 isolates (subset of above described 13 HIV-1 isolates). These samples showed >50% neutralization of 6 and 4 isolates (out of 8) respectively (Fig. 1). These samples showed >30% neutralization (mean+3 SD inhibition shown by plasma from 20 healthy HIV uninfected individuals against one primary HIV-1 isolate) in all (except one) reactions. This suggests that the presence of cross-reactive neutralizing antibody response is not restricted to plasma samples that showed ≥90% neutralization in primary screening.

Analysis of HIV-1 subtype C env (gp120) nucleotide sequences revealed that sequences from India show limited genetic diversity compared to other subtype C prevalent countries

We investigated diversity among HIV-1 *env* (gp120) nucleotide sequences from India, Burundi, Botswana, South Africa, Tanzania and Zambia. The sequences were selected by using "one sequence/patient" mode at the interface of NIH Los Alamos HIV sequence database (http://hiv-web.lanl.gov/components/hiv-db). There were 17 sequences from India, 8 from Burundi, 51 from Botswana, 180 from South Africa, 15 from Tanzania and 10 from Zambia. All sequences from India, Burundi, Tanzania, Zambia and 17 randomly selected sequences from Botswana and South Africa were analyzed for genetic diversity. The pairwise % genetic divergence was determined using MegAlign program (DNASTAR Inc). The mean±SD and median genetic divergence (%) of all pairwise sequence comparisons was 9.9±2.3 and 10.0 for India, 12.1±1.3 and 11.9 for Burundi, 15.0±1.2 and 14.9 for Botswana, 15.2±1.9

Table 1
ID_{50} shown by plasma samples (that showed ≥90% neutralization in primary screening) against thirteen isolates

Sr. No	Sample Id[#]	Gender	CD4 (cells/ mm^3	HIV-1 subtype C primary isolate Year of isolation, Origin													Geometric mean ID_{50} against all isolates
				VB39 1999 MH	VB49[$] 1999 MH[1]	VB37 1999 MH	418201 2004 MH	VB67 1999 KA[2]	VB58 1999 KA	VB85 2000 Delhi	418269 2004 MH	KH4922 2004 MH	26191 2000 MH	VB55 1999 Delhi	VB27 1999 Goa	BH9390 2005 MH	
1	CPI 177	M	97	320*	320*	160*	320*	160*	320*	160*	320*	320*	160*	160*	320*	40*	201.6
2	CPI 220	M	285	320*	120*	320*	320*	320*	320*	160*	160*	160*	160*	160*	320*	40	185.8
3	CPI 267	M	24	320*	120*	160*	320*	160*	160*	160*	320*	160*	160*	80*	320*	80*	165.5
4	CPI 243	M	305	160*	320*	160*	320*	160*	320*	160*	320*	160*	80*	80*	320*	20	160.0
5	CPI 287	M	410	160*	120*	320*	160*	320*	320*	160*	160*	160*	80*	160*	320*	20	156.2
6	CPI 255	F	406	160*	120*	160*	80*	160*	160*	160*	160*	160*	80*	160*	160*	40	124.0
7	CPI 261	M	521	160*	160*	160*	160*	320*	40*	80*	160*	80*	320*	40*	80*	20	100.8
8	CPI 229	F	476	160*	160*	80*	160*	80*	80*	20	40*	40*	160*	40*	40*	10	56.6
9	CPI 91	M	700	80*	160*	80*	20	40*	40*	80*	40	40*	80*	40*	40	<10	51.5
10	CPI 244	M	158	80*	20*	40*	40*	160*	40*	80*	<10	40	40	80*	20	40*	45.4
11	CPI 294	M	82	160*	160*	80*	80*	80*	40*	10	40	40*	80*	40*	40	10	44.9
12	CPI 223	F	516	160*	120*	80*	40*	80*	20	20	160*	40*	40*	40*	10	<10	44.2
13	CPI 212	M	389	40	320*	40*	40	80*	80*	10	40*	10	<10	40*	20	<10	40.0
14	CPI 133	M	224	80*	40*	20	40*	80*	40*	80*	20	20	20	80*	40	10	33.6
15	CPI 214	M	326	320*	40*	40	10	40*	40*	160*	10	80*	80*	40*	10	10	31.7
16	CPI 158	M	95	160*	40*	80*	20	20	20	10	10	20	40*	10	10	<10	20.0
17	CPI 153	M	311	320	160*	10	40	10	20	<10	10	20	10	<10	10	<10	18.5
18	CPI 99	M	48	80*	80*	40	<10	10	40*	<10	<10	<10	10	20	<10	<10	15.9
19	CPI 218	M	8	320	40*	<10	<10	10	<10	<10	10	<10	10	<10	<10	<10	14.1
20	PNP	–	–	<10	<10	<10	10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
Geometric mean ID_{50} shown by all plasma				160.0	106.8	80	76.8	74.4	71.3	61.7	60.1	60.1	58.8	57.7	55.4	17.7	

Shaded box represents ≥50% neutralization. <10 represents <50% neutralization at 1/10 plasma dilution. PNP: Pooled Normal Plasma (negative control).
[#]Plasma samples were collected during year 2001–2003 from Pune (Maharashtra), India.
[$]HIV-1 isolate used for primary screening.
[1]Maharashtra.
[2]Karnataka.
*Plasma–virus reaction where ≥90% neutralization was observed at 1/10 plasma dilution.

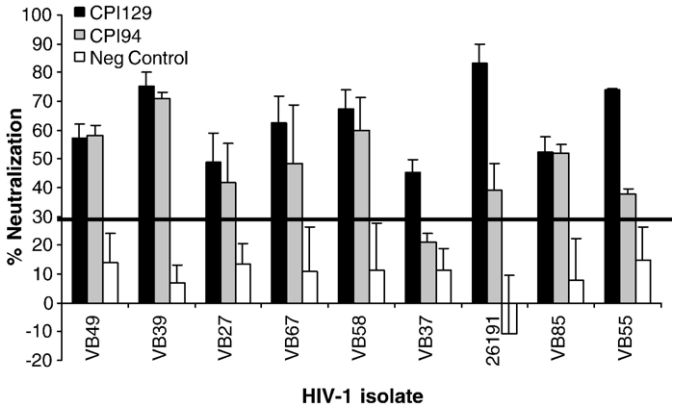

Fig. 1. Cross-reactivity of plasma samples selected for 50–60% neutralizing activity. Two plasma samples (CPI 129 and CPI 94) showing 50–60% neutralizing activity against one primary HIV-1 isolate (VB 49) were tested against panel of primary HIV-1 isolates (*X*-axis). Mean + SD obtained in two independent experiments is presented.

and 15.3 for South Africa, 15.3 ± 1.3 and 14.3 for Tanzania and 14.3 ± 1.7 and 14.2 for Zambia. The divergence observed among sequences from India was significantly low ($p < 0.001$) compared to divergence observed among sequences from other subtype C prevalent countries (Fig. 2).

Discussion

Several studies suggest limited genetic diversity among circulating viruses in India. In phylogenetic analysis, most Indian viruses form compact cluster and probably are descendent of a single founder strain or limited diversity of strains introduced at the beginning of the epidemic in India (Agnihotri et al., 2004, 2006; Eshleman et al., 2005; Gaschen et al., 2002; Gupta et al., 2005; Jere et al., 2004; Kurle et al., 2004; Novitsky et al., 2002; Novitsky et al., 1999; Shankarappa et al., 2001). Constant shifts in glycosylation sites in envelope protein play important role in protecting virus from neutralizing antibodies (Wei et al., 2003). During analysis of envelope protein sequences from India, it was observed that all potential glycosylation sites were well conserved (Agnihotri et al., 2004). Due to high degree of homogeneity in circulating viruses, distinction between autologous and heterologous neutralization may be blurred. This may result into extensive cross-neutralization of heterologous viruses. This speculation provoked to investigate cross-reactivity of neutralizing antibody response of HIV-1 infected individuals from India. Currently, there is paucity of data about neutralizing antibody response in HIV-1 infected individuals from India.

We selected ART-naive plasma samples showing ≥ 90% neutralization of a heterologous, primary HIV-1 subtype C isolate and subsequently tested their ability to neutralize panel of HIV-1 isolates. These isolates were obtained from epidemiologically unlinked HIV-1 infected individuals. The individuals were residents of four different states of India, and the isolates were obtained at different time points (between year 1999 and 2005). This ensured that viruses used in the study are from diverse origin. The samples showing ≥ 90% neutralizing activity in primary screening showed extensive cross-neutralization of multiple isolates (Table 1). The cross-reactivity was not restricted to samples showing ≥ 90% neutralization. The samples showing 50–60% neutralization in primary screening also showed neutralization of multiple isolates from the test panel (Fig. 1). The broad range of ID_{50} values shown by each plasma sample against different viruses (Table 1) indicate that, although there may be shared neutralization determinants, each isolate may also carry a unique set of such determinants. Minor variations in amino acid sequence of neutralization epitopes can influence antibody binding affinity and contribute to variability. Variations in the exposure of neutralization epitopes may also contribute to differences in the outcome of virus–antibody interactions. The differential cross-neutralizing activity also needs to be evaluated in the light of implicit of mixed polyclonal antibody response.

Since *env* gene is most relevant in the context of HIV-1 neutralization, we investigated genetic diversity among nucleotide sequences of *env* gp120 of HIV-1 subtype C viruses from subtype C prevalent countries. Diversity among sequences from India was found to be significantly low ($p < 0.001$) compared to diversity observed among subtype C sequences from Burundi, Botswana, South Africa, Tanzania and Zambia (Fig. 2). This supports our hypothesis that extensively cross-reactive HIV-1 neutralizing antibody response among Indian patients may be attributable to the shared neutralization determinants among circulating viruses in India. However, considering each virus

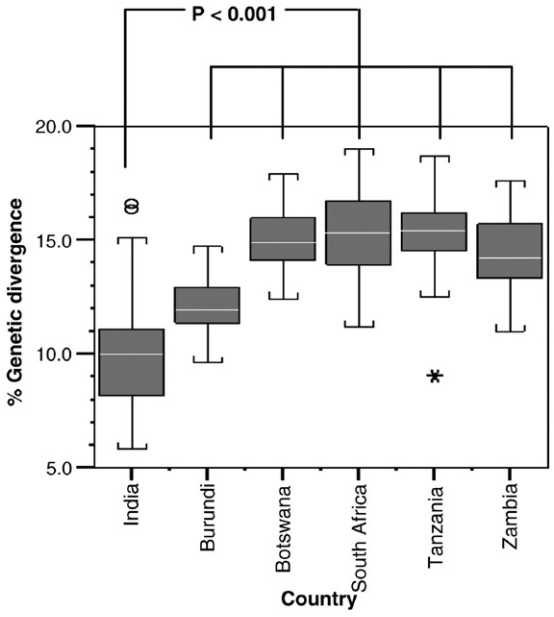

Fig. 2. Diversity among HIV-1 subtype C envelope (gp120) nucleotide sequences from different countries. The sequences from India show significantly less diversity ($p < 0.001$) compared to sequences from other countries. Pairwise % genetic divergence among available nucleotide sequences (one sequence per patient) was determined by MegAlign program. The boundary of the box closest to zero indicates the 25th percentile, a solid line within box shows median and boundary of the box farthest from zero indicates 75th percentile. Whiskers above and below the box indicate the 10th and 90th percentiles. Outliers for India and Tanzania are shown by different symbols.

isolate and plasma showing unique pattern of neutralization, it is necessary that molecular analysis of this cross-reactivity is carried out.

Factors that have been discussed to be positively associated with breadth of HIV-1 neutralizing activity include high $CD4^+$ T cell count (Carotenuto et al., 1998), exposure to multiple viruses due to many partners (Bures et al., 2002) and period after infection (Moog et al., 1997). Out of 19 plasma samples that were tested against a panel of HIV-1 isolates, three were from patients with $CD4^+$ T cell count less than 50 cells/mm^3. Out of these three samples, two showed marginal breadth of neutralization whereas remaining sample showed $\geq$90% neutralization of all thirteen isolates. Three women participants with a history of single constant HIV-1 seropositive partner (monogamous) showed $\geq$90% neutralization of eight or more isolates from the panel indicating that the cross-neutralization may not be just consequence of exposure to diverse virus strains that may occur in multiple partner setting. These findings discount the possibility that the above mentioned factors have overwhelmingly contributed to our interpretations. Date of infection is unknown for all plasma donors. This limitation makes it difficult to interpret whether cross-reactive neutralizing antibodies developed along with response to autologous virus following primary infection or accumulated over time as reported earlier. A report on recent seroconverters from India showed that plasma samples obtained within 1 year of seroconversion mediate neutralization of heterologous HIV-1 isolates (Kulkarni, 2005).

It has been reported that there is selective transmission of neutralization sensitive viruses (Derdeyn et al., 2004). However, the isolate 26191 that was obtained from recently infected individual (within 1 month after seroconversion) did not show more neutralization sensitivity compared to other viruses that were obtained from different stages of infection.

Earlier studies based on neutralization of heterologous HIV-1 primary isolates obtained from same cohort or from same geographic region show that extensive cross-neutralization of such isolates is exclusive feature of Long Term Non-Progressors (LTNPs) (Cao et al., 1995; Carotenuto et al., 1998; Pilgrim et al., 1997; Zhang et al., 1997). Patients with progression of disease show limited cross-neutralization of heterologous isolates after few years of infection (Moog et al., 1997; Wrin et al., 1994). These studies are from countries where HIV-1 B is the predominant subtype. A study of recently subtype C infected individuals from South Africa showed that neutralizing antibody response developed against autologous virus can also neutralize heterologous South African subtype C isolates (Bures et al., 2002). This may be due to shared neutralization determinants among circulating subtype C viruses in South Africa. This is supported by the observation that subtype C viruses show less genetic diversity compared to subtype B viruses (Gaschen et al., 2002). HIV-1 infected individuals from India showed extensive cross-neutralization of circulating subtype C viruses from India. The degree of cross-neutralization and mean neutralization titer was higher compared to study based on subtype C infected individuals from South Africa. This might be due to significantly less diversity observed in subtype C sequences from India compared to South Africa (Fig. 1).

Selection of viruses for evaluation of neutralizing antibody responses induced by various immunogens is a vital concern. Due to limited genetic diversity, a panel of few immunologically assorted HIV-1 isolates might be adequate for assessment of neutralizing antibody responses elicited by various immunogens. Moreover, limited HIV-1 genetic diversity and broad cross-reactivity of neutralizing antibodies generated in HIV-1 infected individuals from India suggest advantages that may be explored further for development of prophylactic HIV-1 vaccine candidates. Immunogen based on the virus that can elicit potent and broadly cross-reactive neutralizing antibody response might be a good candidate for further evaluation. Similarly HIV-1 isolate that is amenable to neutralization by majority of sera indicating presence of consensus neutralization determinants of circulating viruses can be used for development of new immunogens. Immune responses induced by such immunogens might be effective against majority of circulating viruses.

Analysis of molecular basis of the observed extensive cross-neutralization of subtype C viruses from India and investigation of immunogenic properties of these viruses may yield information that could be useful in HIV-1 vaccine development in a scenario of limited genetic diversity.

Materials and methods

Study subjects, blood sample collection and processing

Two hundred and thirty five HIV-1 seropositive individuals (150 male and 85 female) enrolled in a study 'Clinical Progression of HIV Infection', at National AIDS Research Institute, Pune, India were included in the study. HIV seropositivity was determined by commercial solid phase combined ELISA for anti-HIV-1 and anti-HIV-2 antibodies (Innotest HIV-1/HIV-2 Ab Immunogenetics, N.V. Zwijnarde, Belgium; Genetic Systems HIV-1/HIV-2 EIA Sanofi Diagnostics Pasteur, France; UBI HIV-1/HIV-2 EIA, United Biomedical Co. Beijing, China), followed by a rapid test (HIV SPOT, Genelabs Diagnostics, Singapore; HIV TRIDOT Biotech Inc, Parwanoo, India) for all the samples that were positive in the combined ELISA. All patients were HIV-1 infected for unknown duration. Whole blood was collected in EDTA vacutainers at the enrollment visit after obtaining written informed consent. Plasma was aliquoted and stored at −70 °C until tested. The study was approved by Ethics committee of the Institute.

HIV-1 isolates

Thirteen primary HIV-1 subtype C isolates were obtained from virus repository of National AIDS Research Institute, Pune, India. Stocks of all viruses were developed using PBMCs from a single donor. None of the virus was cultured more than three times. Isolates were obtained from individuals that were epidemiologically unlinked. The individuals were residents of

four different states of India (Maharashtra, Goa, Karnataka, Delhi) and were isolated at different time point (from year 1999 to 2005). The isolates from Maharashtra and Karnataka were from different cities within these states. It was not possible to determine time of infection in all patients except the isolate 26191, which was obtained within 1 month of seroconversion from a male patient.

Estimation of $CD4^+$ T cell count

$CD4^+$ T lymphocyte percentages in freshly collected blood samples were determined by flow cytometer using SimulSET software (FACSort; Becton Dickinson Immunocytometry Systems, San Jose, CA) after staining with phycoerythrin labeled anti-CD4 antibody. Absolute $CD4^+$ T cell counts were calculated on the basis of total and differential blood cell counts obtained by using automated hematology analyzer (Sysmex KX 21).

Primary screening for detection of neutralizing antibody response

Neutralization assay was performed using $CCR5^+$ GHOST cells that express Green Fluorescence Protein (GFP) under control of HIV-1 Tat as described by Trkola et al. (1998). In brief, virus (predetermined dilution that produces ~1000 fluorescent cells at the end of the assay)–plasma mixture was incubated at 37 °C for 1 h and was added to GHOST cells in the presence of DEAE–dextran (8 μg/ml). The plate was incubated at 37 °C with 5% CO_2 and humidified atmosphere for 4 days. After incubation, cells were harvested and analyzed on flow cytometer (FACSort; Becton Dickinson). An electronic gate was formed using forward and side scatter and within the gate 15,000 cells were acquired. Number of fluorescent cells was determined after setting quadrants based on cells from uninfected well. All plasma samples were heat-inactivated at 56 °C for 30 min before use. In primary screening, all plasma samples ($n=235$) were tested at 1/10 dilution in duplicate against a primary HIV-1 isolate, VB 49.

Plasma samples from 20 healthy, HIV seronegative individuals were also tested for background response at the same dilution. Background response was 5.8±8.3% (mean±SD).

Neutralizing activity against a panel of primary isolates

Nineteen plasma samples that showed ≥90% neutralization (≥1 log reduction in infectivity) against VB 49 in primary screening were tested against a panel of 13 primary HIV-1 isolates (including the isolate used in primary screening). Six two-fold dilutions of each plasma sample (from 1/10 to 1/320) were tested against each virus from the panel.

Additionally two plasma samples that showed 50 to 60% neutralization in primary screening were randomly selected and were tested against a panel of 8 primary HIV-1 isolates (subset of above 13 isolates) at 1/10 dilution.

Pool of plasma samples from 12 healthy, HIV seronegative individuals was used as a negative control and was tested at 1/10 dilution in each assay. The background response was <30% in all assays.

Diversity among HIV-1 subtype C nucleotide sequences

HIV-1 subtype C gp120 nucleotide sequences from India, Burundi, Botswana, South Africa, Tanzania and Zambia were obtained from NIH Los Alamos HIV sequence database (http://hiv-web.lanl.gov/components/hiv-db). When multiple sequences from same patient were available, one sequence per patient was selected by using "one sequence/patient" mode at search interface of HIV sequence database. There were 17 sequences from India, 8 from Burundi, 51 from Botswana, 180 from South Africa, 15 from Tanzania and 10 from Zambia. All sequences from India, Burundi, Tanzania, Zambia and 17 randomly selected sequences from Botswana and South Africa were analyzed for genetic diversity. The pairwise % genetic divergence was determined using MegAlign program (DNASTAR Inc). The % genetic divergence among sequences from India was compared with divergence among sequences from Burundi, Botswana, South Africa, Tanzania and Zambia by Student's *t*-test.

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